BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

STIC-ILL

GMC
RBI-A4

From: Canella, Karen
Sent: Wednesday, May 14, 2003 3:05 PM
To: STIC-ILL
Subject: ill order 09/230,955

Art Unit 1642 Location 8E12(mail)

Telephone Number 308-8362

Application Number 09/230,955

1. American Journal of Pathology:
   1993 Feb, 142(2):403-412
   1993, 143(4):1150-1158
   1984, 114(3):454-460
   1996, 148(3):865-875
   1965 Sep, Vol. 44, pp. 280-282

2. Cancer Research, 1993 May 15, 53(10 suppl):2287-2299

3. Cancer epidemiology, biomarkers and Prevention, 1996 Jul, 5(7):549-557

4. Lab Investigation:
   1980, 42(1):91-96
   1988, 58(2):141-149

5. Gynecol Oncol, 1982, 13(1):58-66

6. International Journal of Gynecological Pathology:
   1985, 4(4):300-313
   1986, 5(2):151-162
   1992, 11(1):24-29

7. Differentiation:
   1986, 31(3):191-205
   1988, 39(3):185-196

8. Cancer (Phila), 1989, 63(7):1337-1342

9. Cancer Res, 1990, 50(16):5143-5152

10. Virchows Arch B Cell Pathol Incl Mol Pathol, 1987, 54 (2):98-110

11. Acta Histochemica et Cytochemica:
    1994, 27(3):251-257
    1996, 29(1):51-56

12. Archives of Gynecology and Obstetrics, 1989, 246(4):233-242

13. Clin Lab Med, 1995 Sep, 15(3):727-742

14. Clin Obstet Gynaecol, 1984 Apr, 11 (1):5-23

American Journal of Pathology, Vol. 148, No. 3, March 1996

Expression of Ep-CAM in Cervical Squamous Epithelia Correlates with an Increased Proliferation and the Disappearance of Markers for Terminal Differentiation

Sergey V. Litvinov, Willemien van Driel, Connie M. van Rhijn, Hellen A. M. Bakker, Han van Krieken, Gert J. Fleuren, and Sven O. Warnaar

From the Department of Pathology, Leiden University, Leiden, The Netherlands

Ep-CAM, an epithelial adhesion molecule, is absent in normal squamous epith · · but can be detected in some squamous carcinomas. Using a panel of monoclonal antibodies to keratinocyte differentiation and proliferation markers, we investigated the association of Ep-CAM expression with differentiation-related and/or neoplastic changes in cervical epithelium. Normal endocervical glandular epithelium (both columnar and reserve cells) appeared strongly positive for Ep-CAM, whereas ectocervical squamous epithelial cells did not express this molecule. Expression of Ep-CAM (in basal cells) was sometimes observed in morphologically normal ectocervical tissue but only in areas bordering cervical intraepithelial neoplasia (CIN) lesions. At the early stages of neoplasia the expression of Ep-CAM was regularly present in squamous epithelium, in general consistent with the areas of atypical, undifferentiated cells. Thus, in CIN grades I and II, the basal/suprabasal layers of the epithelia were positive, whereas in CIN grade III lesions, up to 100% of the cells, over the whole thickness of the epithelium sometimes excluding the very upper layers, expressed Ep-CAM. A clear increase, not only in number of positive cells but also in levels of Ep-CAM expression (intensity) was observed during progression from CIN I to CIN III. Expression of Ep-CAM in ectocervical lesions did not coincide with a reappearance of the simple epithelium cytokeratins (CK8 and CK18). On the other hand, expression of Ep-CAM in atypical cells of CIN lesions correlated with the disappearance of CK13, which normally marks cells undergoing squamous differentiation. As was shown with Ki-67, a marker for proliferating cell populations, the areas of Ep-CAM expression were also the areas of enhanced proliferation. Cells expressing Ep-CAM did not express involucrin, a marker for cells committed to terminal differentiation. In the majority of both squamous and adenocarcinomas of the cervix a strong expression of Ep-CAM was observed, although some decrease in the expression (both the intensity and the number of positive cells), as compared with CIN III lesions, was observed in the areas of squamous differentiation. This study demonstrates that the expression of Ep-CAM in cervical squamous epithelium is associated with abnormal proliferation of cell populations that are not committed to terminal differentiation. (Am J Pathol 1996, 148:865–875)

The 40-kd epithelial protein, Ep-CAM, encoded by the GA733-2 gene,[1] when expressed in cells deficient in intercellular adhesion, demonstrates the characteristics of a homophilic cell-cell adhesion molecule.[2] The exact role of this molecule in epithelial tissues is not clear yet. However, we have demonstrated that Ep-CAM is of importance for intercellular interactions of carcinoma cells that have decreased levels of cadherins,[3] which suggests that Ep-CAM is an adhesion molecule contributing to intercellular interactions, at least in some tissues.

Supported by a research grant from the Dutch Cancer Society/ Queen Wilhelmina Fund (RUL 94-1107) and a research grant from Centocor Inc., Malvern, PA.

Accepted for publication December 6, 1995.

Address reprint requests to Dr. S. V. Litvinov, Department of Pathology, Leiden University, Building 1, L1-O, P.O.Box 9600, 2300 RC Leiden, The Netherlands

In normal human tissues Ep-CAM is expressed in most simple, columnar, and pseudostratified epithelia[4] but is absent in squamous stratified epithelial cells. However, during carcinogenesis a *de novo* expression of Ep-CAM can be observed in squamous tissues.[5-7] Thus, Ep-CAM is expressed in squamous carcinomas of the bronchus, head and neck region, and cervix but not in squamous carcinomas of the skin, although it can be abundantly expressed in basal cell carcinomas.[6,7] As normal differentiation of cells is disturbed in the majority of carcinomas of squamous origin, and expression of histological markers typical for simple epithelia may occur in squamous carcinoma cells,[8,9] we have investigated whether the appearance of Ep-CAM in squamous epithelial neoplasias correlates with the expression of markers for simple epithelia and is connected to proliferative and differentiation-related changes of squamous epithelial cells.

In the uterine cervix the sequential morphological steps of tumorigenesis can be identified, with a progression from low to high grade squamous intraepithelial lesions and, ultimately, to invasive carcinomas.[10,11] Therefore, studies in uterine cervix allow us to follow the expression of Ep-CAM in correlation with the changing pattern of differentiation markers[9,12] in progressing neoplasia.

Our results showed that the expression of Ep-CAM in squamous cervical epithelium correlates with an enhanced proliferative activity and with the loss of tissue-specific markers, including markers for terminal differentiation of squamous epithelial cells.

Materials and Methods

Tissues and Pathological Evaluation

The frozen tissue specimens used in this study comprised biopsies or surgical resection specimens of lesions of the uterine cervix. A total of 42 specimens representing normal, metaplastic epithelia, and three grades of cervical intraepithelial neoplasia (CIN) were studied. In many samples, normal epithelium and metaplasia or intraepithelial neoplasia were found within the same specimen. Normal cervical epithelial tissues were identified in 14 tissue specimens, along with 28 cases of squamous metaplasia and 39 cases of CIN. The CIN cases were subdivided into low grade squamous intraepithelial lesions comprising the cases of CIN I, and high-grade lesions, within which we discriminated CIN II and CIN III lesions. Additionally, sections of 15 cervical squamous carcinomas and 10 cervical adenocarcinomas were investigated.

Sections, subsequent to those used for immunohistological staining, were stained with hematoxylin and eosin and analyzed by two independent pathologists with respect to the type and character of the lesions identified within the tissue sample.

Antibodies

Antibodies to cytokeratins CK5 (clone AE14), CK10 (clone RKSE60), CK14 (clone RSK107), and CK16 (clone LL025) were kindly provided by Prof. F. Ramaekers. Antibodies to cytokeratins CK7 (clone C-68), CK8 (clone H1), CK13 (clone 1C7), CK17 (clone E3), and CK18 (clone DC-10) were obtained from Thamer Diagnostica. All anti-cytokeratin antibodies used are well characterized with respect to their specificity and recognize single-type cytokeratins. The anti-Ep-CAM antibody 323/A3[13] was kindly provided by Centocor (Malvern, PA). Antibody Ki-67 to a proliferation marker was obtained from Boehringer Mannheim (Mannheim, Germany). Antibody to involucrin (clone SY5) was obtained from Sigma Immunochemicals. As negative control, monoclonal antibodies (MAbs; of IgG1 and IgG2a isotypes) to episialin, a protein that is not expressed in squamous epithelial tissue, were used.

Immunohistochemistry

Cryostat sections were fixed in cold methanol and then rinsed with acetone and air dried. Sections were preincubated for 30 minutes with either 10% normal goat serum (for immunohistochemistry) or with 5% skimmed milk solution (for immunofluorescent staining). Both solutions were prepared in phosphate-buffered saline. Immunohistochemical staining with 323/A3 MAb was performed as described previously.[13] For double immunofluorescent staining for Ep-CAM and an additional marker, primary antibodies (all of IgG1 isotype, except anti-CK17, E3, which is IgG2b, and anti-CK14, RSK107, which is IgG3) were applied in a mix with the 323/A3 MAb (IgG2a). The reacted antibodies were detected using an anti-mouse IgG1 (or, respectively, IgG2b or IgG3)-fluorescein isothiocyanate conjugate in a mix with an anti-mouse IgG2a-Texas-Red conjugate. All conjugates were obtained from Southern Biotechnology (Birmingham, AL). The immunofluorescent staining was analyzed using the BRC-600 confocal microscope (Bio-Rad, Richmond, CA).

Each of the tissue samples was analyzed with all of the markers used. The specificity of the staining was verified by using a control antibody of the same IgG isotype. For double-fluorescence analysis the

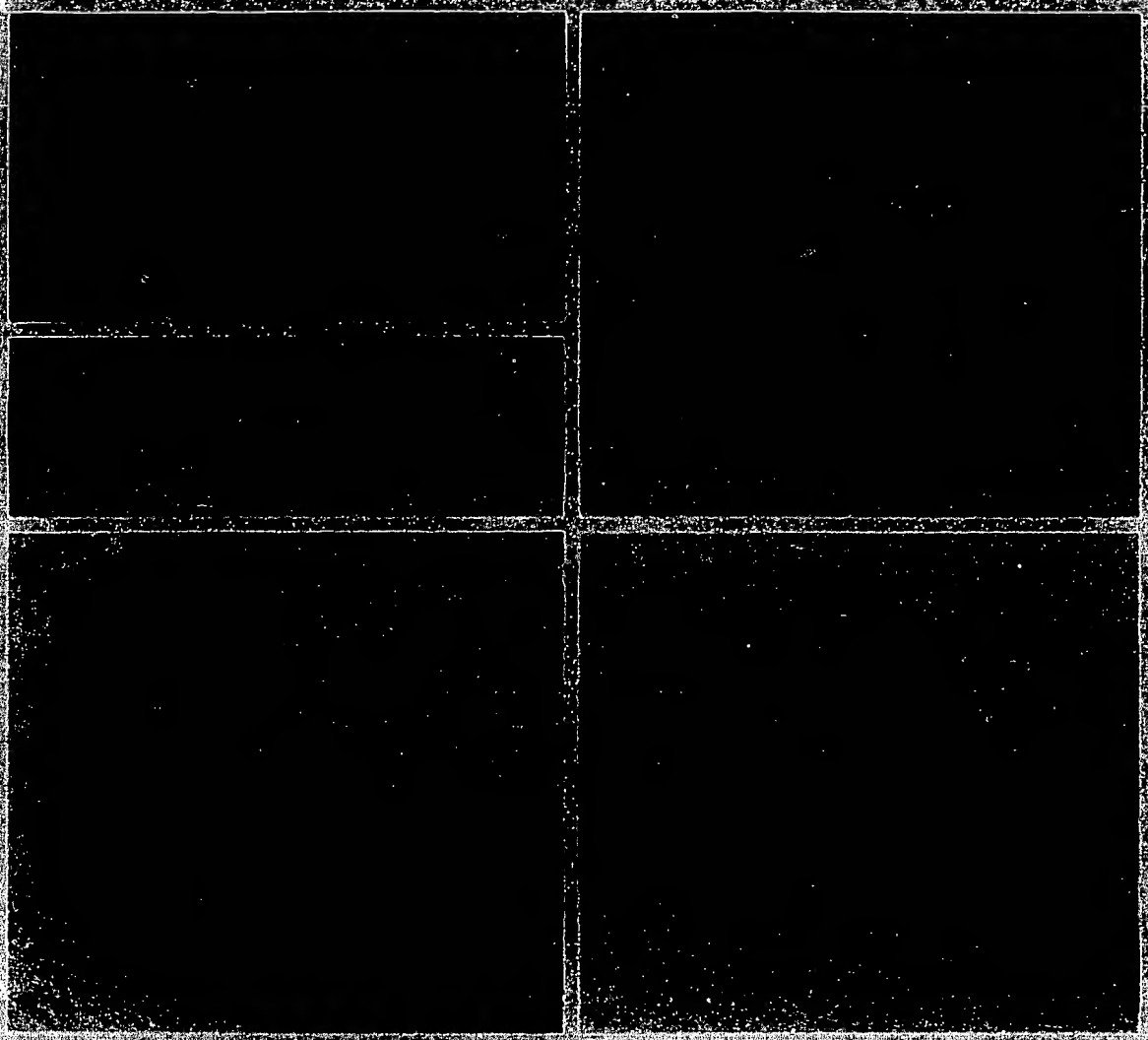

Figure 1. *Expression of Ep-CAM in normal, dysplastic, and neoplastic tissue of uterine cervix, as detected by indirect immunohistochemistry using the 323/A3 MAb. A: Normal cervical squamous epithelia. B: Normal glandular epithelium. C: Immature metaplasia with signs of squamous differentiation. D: Low grade squamous intraepithelial lesion. E: High grade squamous intraepithelial lesion.*

absence of the leaking of one type of fluorescence into another was confirmed by using cultured epithelial cells stained with antibodies to two markers with completely nonoverlapping patterns (Ep-CAM, which is located at the cell-cell boundaries and in cytoplasm, and Ki-67, which is located in the nucleus only).

Results

Expression of Ep-CAM in Normal, Dysplastic, and Malignant Cervical Epithelium

Immunohistochemistry on cervical tissues showed that normal ectocervical epithelium was negative for Ep-CAM expression (Figure 1A), in contrast to normal endocervical glandular epithelium, both columnar and reserve cells (Figure 1B), which was highly positive for Ep-CAM. In the transformation zone, a clear border between Ep-CAM-positive endocervical epithelium and negative cells of squamous differentiation was observed.

In immature and mature squamous metaplastic epithelia, a strong expression of Ep-CAM was found; the intensity of staining of the majority of cells in immature metaplastic lesions was comparable to the staining of columnar endocervical epithelium. In mature squamous metaplasia, the areas of squamous differentiation showed a decrease in Ep-CAM expression up to complete negativity (Figure 1C).

Low expression of Ep-CAM was occasionally detected in basal/suprabasal layers of morphologically normal squamous epithelium bordering intra-

epithelial neoplasia. At higher levels, the expression of Ep-CAM was detected in CIN I, with the intensity increasing in the higher grade neoplastic lesions (Figure 1, D and E). The areas of Ep-CAM expression were clearly overlapping, if not completely identical, with the areas of atypical/undifferentiated cells, with the marker disappearing in the upper, more differentiated layers of the epithelia. Expression of Ep-CAM was found in all CIN lesions analyzed.

Ep-CAM expression was detected in all squamous carcinomas analyzed as well as in all adenocarcinomas. In some squamous carcinomas, only a low level of Ep-CAM expression was detected (not shown); in all cases it was related to a pronounced squamous differentiation in these lesions.

Expression of Ep-CAM and Cytokeratins in Squamous Metaplastic Epithelia

Reserve cells are able to differentiate into both simple and squamous epithelia, and they express a mixed pattern of cytokeratins typical for both types of epithelia.[14,15] The cytokeratin markers, typical for differentiated squamous, columnar cells and reserve cells as observed in the previous studies,[15] and confirmed in the current study, are shown in Figure 2. Using double staining with anti-Ep-CAM MAb combined with MAbs to CK7 (columnar cells only) and CK18 (columnar and reserve cells), we demonstrated that Ep-CAM is expressed in reserve cells as well as in columnar cells.

There was no correlation between the expression of Ep-CAM and reserve cell cytokeratins (CK14 and CK17) during epithelial transdifferentiation. Thus, immature squamous metaplasias were strongly positive for Ep-CAM, although only a subpopulation of cells in these dysplasias, mainly the cells located in the basal layer, were expressing CK14 and CK17 (Figure 3). In mature metaplasia, the expression of Ep-CAM was disappearing in areas of squamous differentiation but was still observed more widely than CK14 and CK17.

The expression patterns of Ep-CAM and of the simple epithelial cell cytokeratins (CK8 and CK18) were identical in metaplastic tissue (Figure 4, A and B). However, the expression of CK13, a squamous differentiation-related cytokeratin, was observed mainly in Ep-CAM-negative regions of mature squamous metaplasia (Figure 4, C and D). The results are summarized in Figure 2.

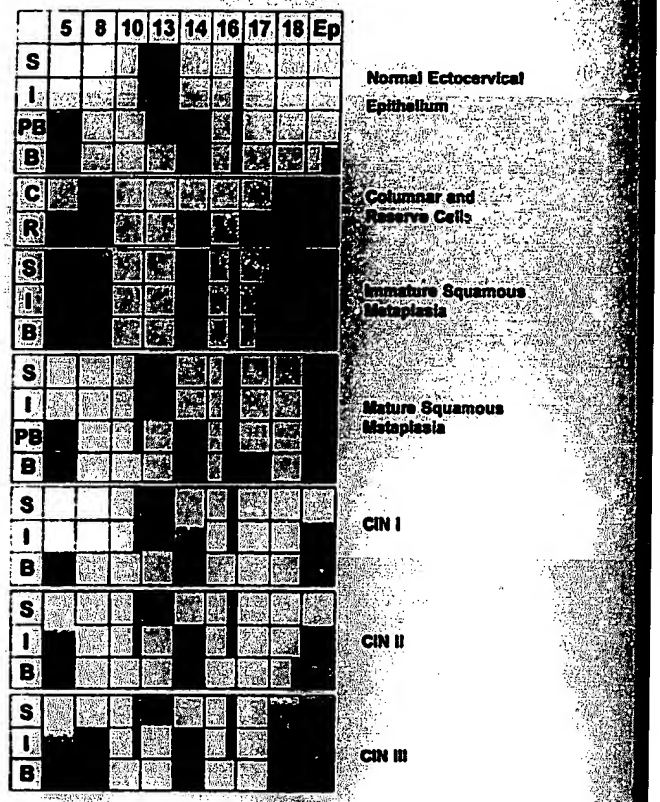

Figure 2. *Schematic representation of cytokeratin and Ep-CAM expression patterns as observed in normal cervical, metaplastic, and neoplastic cervical tissues. The data were obtained on the basis of immunohistochemical investigation of 42 specimens of cervical epithelial tissues (see Materials and Methods). The human cytokeratins are indicated by their respective numbers. Black, gray, or white filling of the bars indicates, respectively, strong, moderate, or no expression of the marker. Gradual filling of the bar indicates gradual decrease or disappearance of the marker in the respective layers of epithelia. A completely filled bar indicates the presence of the marker at the indicated intensity in 75 to 100% of the normal lesion tissue specimens. A one half or one quarter filled bar indicates the presence of the marker in 25 to 50 or less than 25% of cases, respectively. B, basal cells; PB, parabasal cells; I, cells of the intermediate layer; S, superficial cells; R, reserve cells; C, columnar cells.*

Expression of Ep-CAM and Cytokeratins in CIN Lesions

Expression of Ep-CAM at early stages of dysplasia/neoplasia in cervical epithelia was observed in all basal and parabasal cells, but only in basal cells was it co-expressed with CK5. All CK5-positive cells were also Ep-CAM positive in all CIN I and II lesions, but usually a few additional upper layers of cells were positive for Ep-CAM. In a majority of CIN III lesions, no CK5 expression was detected; occasionally, in some lesions, it was limited to a few solitary cells. In contrast, almost all cells in CIN III lesions were strongly expressing Ep-CAM (Figure 5).

CK14 was present in basal and suprabasal cells of ectocervical epithelia, although the intensity of its expression varied greatly, and it was not as regularly

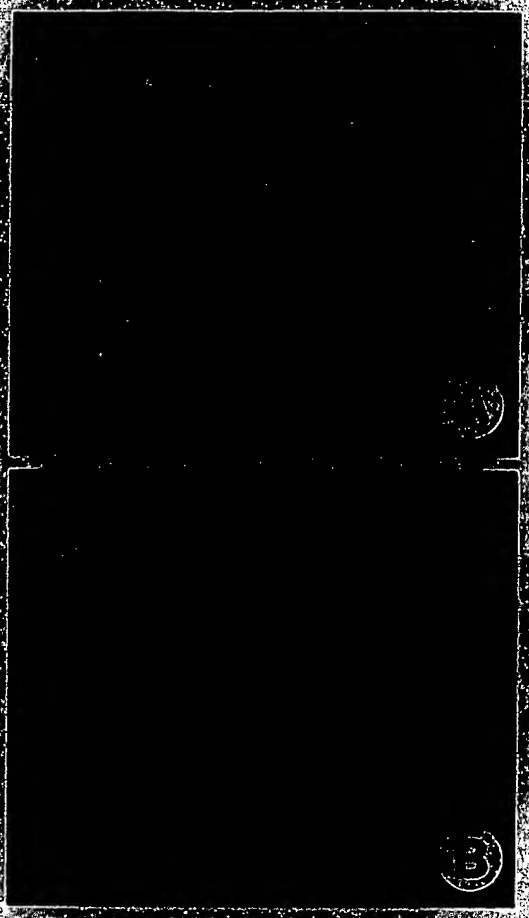

Figure 3. Expression of cytokeratin 17 (green) and Ep-CAM (red) in immature (A) and mature (B) squamous metaplasia. Yellow marks the areas of co-expression of both markers.

present a cytokeratin as CK5. In several CIN lesions, where CK14 was detected, the expression of the latter was, similar to CK5, associated with the more basal compartments of CIN, and all CK14-positive cells in lesions were also Ep-CAM positive. However, in the course of differentiation of dysplastic cells toward the squamous phenotype, CK14 was disappearing earlier than Ep-CAM (Figure 6). The CK17-positive groups of cells were observed occasionally in some CIN III lesions but in a pattern that was much more limited than that for the expression of Ep-CAM. In general, no correlation between expression of Ep-CAM and the markers of reserve cells (CK14 and CK17) was observed, as was true for other occasionally observed cytokeratins, such as CK10 and CK16.

Expression of CK10 was observed very rarely in the CIN lesions, usually in the upper layers of squamous epithelia. Where observed, the CK10 has a pattern of expression complementary to Ep-CAM (not shown). Expression of CK16, which is supposed to be associated with an enhanced proliferative activity,[9] was observed in a very limited number of lesions, usually in the suprabasal areas of CIN, with no correlation to Ep-CAM expression (see Figure 2 for the summary of results).

Expression of simple epithelial cytokeratins CK8 and CK18 was observed in CIN III lesions only. Therefore, the expression of Ep-CAM, which also occurs in CIN I and CIN II, was not related to the expression of these differentiation markers (not shown).

In normal squamous epithelia, CK13 appears already in parabasal cells, with expression increasing toward the upper epithelial layers (Figure 7A). However, in premalignant intraepithelial lesions, in those areas where expression of Ep-CAM was found, the border of CK13 expression was moved to the upper layers. In most dysplastic lesions and intraepithelial neoplasia, the pattern of CK13 is complementary to the pattern of Ep-CAM. Only in a few CIN III lesions were the patterns of these two markers observed to partially overlap, although the Ep-CAM-positive cell layers were mainly negative for CK13, and the CK13-positive cells were largely located superficially to the Ep-CAM-positive cells (Figure 7, B–D).

Expression of Ep-CAM and Markers for Terminal Differentiation

We observed that at very early stages of dysplasia in the cervix the expression of Ep-CAM in para- and suprabasal layers of squamous epithelia was accompanied by the disappearance of CK13, which marks all cells entering the terminal squamous differentiation. Therefore, we investigated the co-expression of Ep-CAM and involucrin, a marker for terminal differentiation of squamous epithelia,[16] in normal squamous and dysplastic cervical epithelia.

In normal ectocervical epithelia, the expression of involucrin occurred in the suprabasal layers and increased toward the upper layers. In tissues where expression of Ep-CAM was observed, involucrin expression occurred only in cells negative for Ep-CAM. In CIN I and CIN II lesions, where Ep-CAM was expressed in basal layers and gradually disappeared toward the upper layers of the stratified epithelium, involucrin appeared only when the cells became negative for Ep-CAM. Remarkably, in most cases we studied, at least one layer of cells negative for both markers separated the Ep-CAM-positive layers from those expressing involucrin (Figure 7, E–H). In particular, it also suggests that in cells of intraepithelial neoplasia the expression of CK13 precedes the expression of involucrin (compare the staining for these markers in Figure 7).

Figure 4. *Patterns of Ep-CAM expression and squamous epithelial cytokeratin 13 and simple epithelial cytokeratin 18 in metaplastic tissue.* B *and* D: *Ep-CAM.* A: *Cytokeratin 18.* C: *Cytokeratin 13.* A *and* B: *Immature squamous metaplasia.* C *and* C: *Mature squamous metaplasia.*

Expression of Ep-CAM and Cell Proliferation in Intraepithelial Lesions

As can be concluded from the data above, cells expressing Ep-CAM in CIN lesions do not commit to terminal differentiation. Therefore, we expected them to be in a proliferative state. Ki-67 marks all cells outside the G_0 phase, increasing from the G_1 phase to the M phase of the cell cycle.[17] Only solitary Ki-67-positive cells were observed in both columnar and squamous normal epithelia (in the basal layer only). In contrast, an active proliferation, as reflected by a high percentage of Ki-67-positive cells, was observed in squamous metaplasia, where the expression of Ki-67 (areas containing the positive cells) was overlapping with areas of Ep-CAM expression (Figure 8A). Similarly, double staining for Ep-CAM and Ki-67 in squamous cervical tissues and neoplasia showed completely overlapping expression patterns for these markers (Figure 8, B and C).

Discussion

The epithelial cell adhesion molecule Ep-CAM is expressed in most simple, pseudostratified, and transitional epithelia but is absent in adult squamous epithelial tissues. However, a large percentage of squamous cell carcinomas[5] (excluding those originating from the skin[6]) do express Ep-CAM. Cervical squamous epithelium is ideally suited to study the relation of Ep-CAM expression to the neoplastic process, as a number of stages of gradually progress-

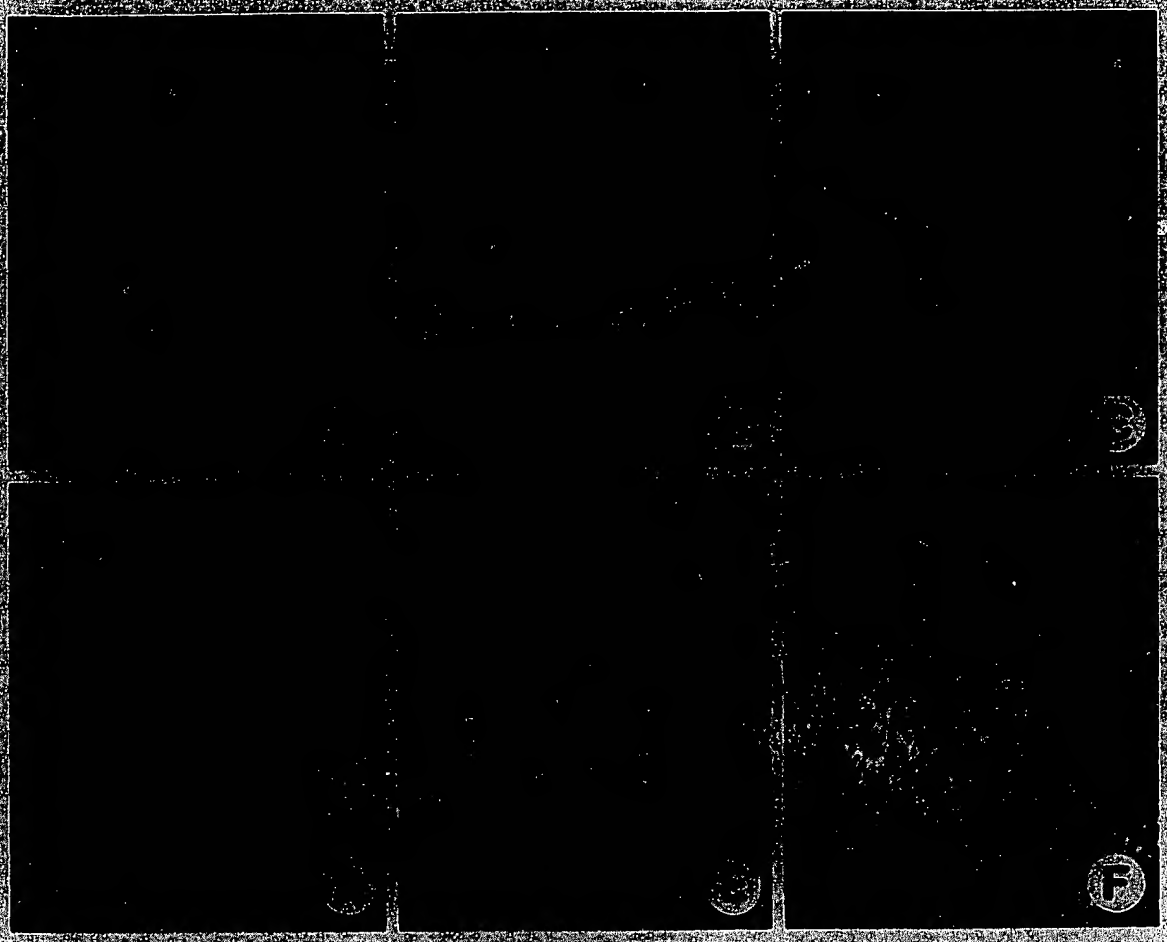

Figure 5. *The patterns of expression for Ep-CAM (A to C) and cytokeratin 5 (D to F) in a low grade squamous lesion (A and D), in CIN II (B and E), and in CIN III (C and F).*

ing neoplastic changes can be distinguished in this tissue.

Normal squamous epithelium of the ectocervix is negative for Ep-CAM, although a very low level of its expression can be detected in the basal cell layer in some tissue specimens. In CIN lesions, the expression of Ep-CAM was found in basal and suprabasal cell layers (summarized in Figure 2), increasing progressively from CIN I to CIN III. Usually the pattern of Ep-CAM expression was clearly overlapping with the undifferentiated cell population in CIN lesions.

Despite the fact that in normal tissue no detectable levels of Ep-CAM are expressed, in dysplastic squamous epithelium, which can be viewed as the earliest stage of neoplastic change, Ep-CAM is clearly co-expressed with the basal cell cytokeratins CK5 and CK14, which mark the proliferating cell population. In contrast, the expression patterns of Ep-CAM and CK13, a marker for squamous differentiation, are complementary, and in CIN lesions the expression of CK13 was observed only in the upper layers of epithelial cells, from suprabasal to superficial layers depending on the CIN grade, where the expression of Ep-CAM was decreasing or disappearing. Expression of CK13 suggests that the Ep-CAM-negative cells in CIN lesions were still undergoing a tissue-specific terminal differentiation. This was confirmed, as all Ep-CAM-negative cells did express involucrin, a marker for terminal differentiation of keratinocytes. In CIN I to CIN III lesions the appearance of this marker was observed only after the complete disappearance of Ep-CAM from the cells. Additional analysis of CIN tissue using Ki-67, a marker for all proliferating cells outside the G_0 phase, confirmed the collateral conclusion that expression of Ep-CAM in ectocervical tissue is associated with an actively proliferating cell population that does not enter terminal differentiation.

The presence of Ep-CAM in basal layers of cervical squamous epithelia seems to reflect an early event in cervical carcinogenesis. Its expression is clearly associated with atypical cells in CIN lesions and increases from low grade to high grade intraepithelial neoplasia. A number of other changes were

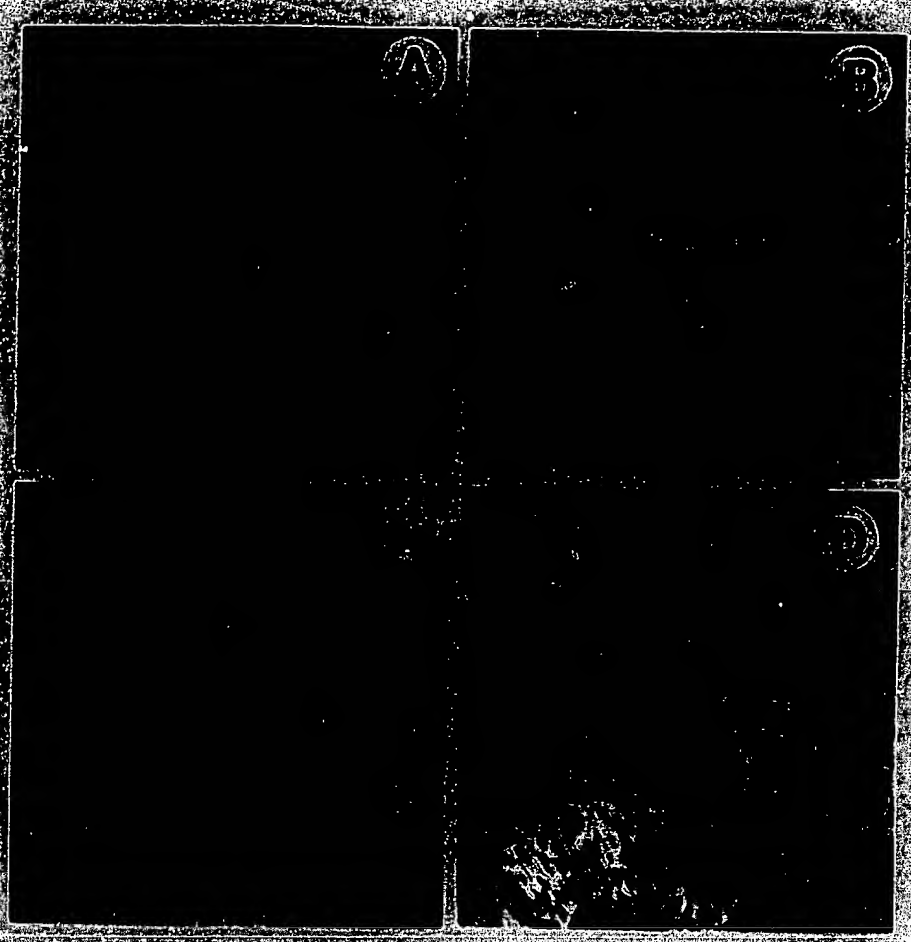

Figure 6. *Expression of cytokeratin 14 (A and C) and Ep-CAM (B and D) in a low grade squamous lesion (A and B) and a high grade squamous lesion (C and D).*

reported in populations of cells in CIN lesions that we found to be positive for Ep-CAM. Thus, the atypical cells in cervical lesions showed a reduced expression of syndecan-1, a proteoglycan that binds growth factors and extracellular matrix components and is associated with normal squamous differentiation of ectocervical cells.[18] The same population of cells was also reported to contain elevated levels of p53,[19] which clearly points to the active proliferation of these cells. Our data confirm a high proliferative activity in undifferentiated cell layers of CIN lesions comparable to that present in carcinomas as reported by Brown et al,[20] also on the basis of Ki-67 antigen expression. Therefore, expression of Ep-CAM in squamous lesions is clearly associated with a crucial disturbance of normal proliferation and differentiation of keratinocytes.

In this respect, the fact that Ep-CAM is expressed in reserve cells, and is highly expressed in immature squamous metaplasia, attracts attention. The reserve cells are capable of differentiating into squamous epithelia both *in vitro* and in nude mice xenografts,[14] which seems to be a direct reflection of the process of epithelial transdifferentiation that occurs *in situ*.[21,22] By transdifferentiation the endocervical simple epithelium is replaced by an immature squamous epithelium that further progresses into mature squamous epithelium.[22] The squamous metaplastic area of the transformation zone is usually the site where squamous intraepithelial lesions and cervical carcinomas develop. It is quite plausible to suggest that expression of Ep-CAM, which normally should be repressed as soon as the transdifferentiating cells acquire the squamous phenotype, continues in dysplastic/neoplastic epithelium.

The observed continuous expression of Ep-CAM in the basal layers of dysplastic lesions may itself be a factor contributing to the disturbances in normal differentiation processes in such lesions.

As was demonstrated by Wheelock and Jensen,[23] E-cadherin has a central role in the regulation of the stratified organization of squamous epithelia, and the disturbance in E-cadherin-mediated junctions re-

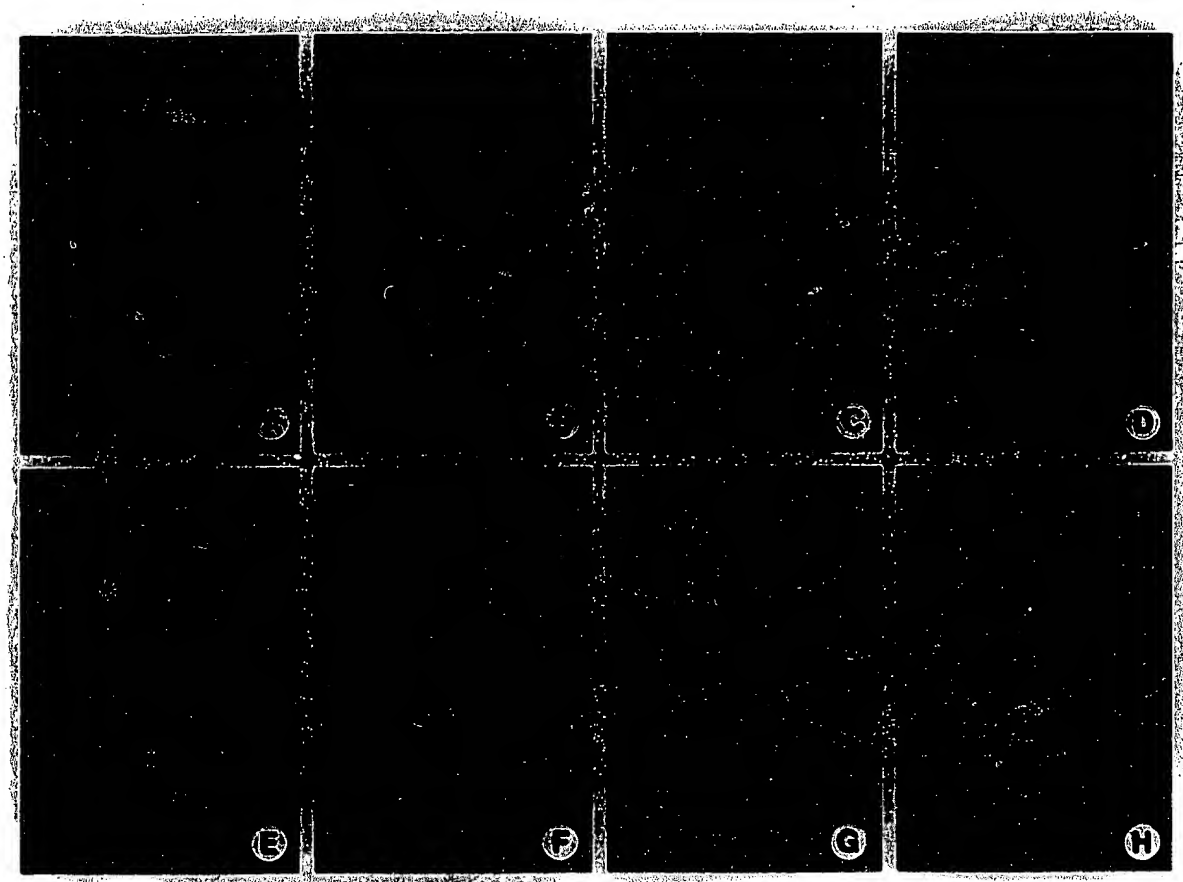

Figure 7. *Expression in squamous tissues of Ep-CAM (red staining) and markers for squamous/terminal differentiation of keratinocytes (green staining).* **A** *to* **D**: *Cytokeratin 13.* **E** *to* **H**: *Involucrin.* **A** *and* **E**: *Normal cervical squamous epithelium.* **B** *and* **F**: *CIN I.* **C** *and* **G**: *CIN II.* **D** *and* **H**: *CIN III lesions.*

sults in abrogation of normal morphogenesis in squamous tissue. Normally, in cells that lose contact with the basal membrane and move to the upper layers (para- and suprabasal), an increased expression of E-cadherin is observed, accompanied by an expression of markers for keratinocyte terminal differentiation.[24] *In vitro*, addition of anti-E-cadherin antibody capable of dissociating the cadherin-mediated intercellular junctions disturbs the calcium-induced stratification of keratinocytes.[23] Recently we have demonstrated that in L cells transfected with E-cadherin cDNA, and supertransfected with the Ep-CAM cDNA, an increase of Ep-CAM expression results in a decrease of E cadherin-mediated cell-cell interactions and in an increased proliferative activity of cells (Litvinov et al, submitted for publication). Ep-CAM expression, therefore, is capable of affecting negatively the cadherin-mediated junctions, and

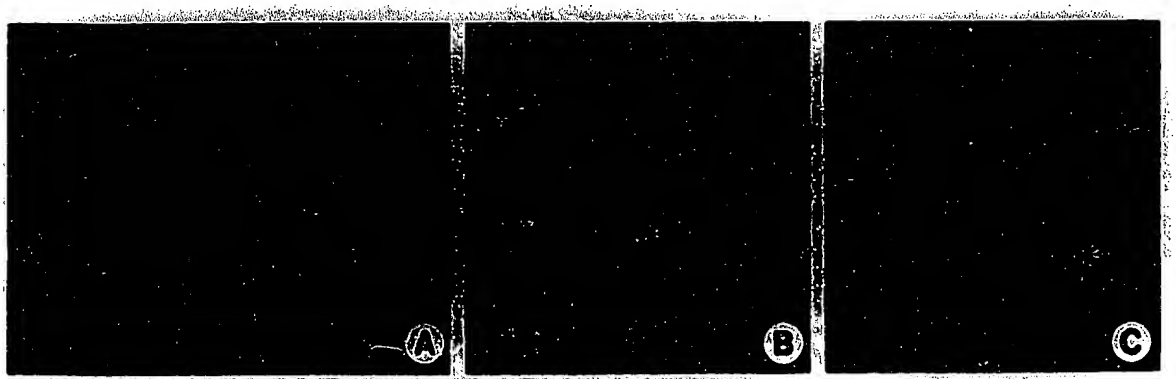

Figure 8. *Correlation of Ep-CAM expression with cell proliferation. A double staining with anti-Ep-CAM MAb (red) and Ki-67 MAb (green nuclear staining) was performed for tissues of squamous metaplasia* (**A**), *low grade squamous lesion* (**B**), *and high grade squamous lesion* (**C**).

this observation can be extrapolated to the discussed neoplastic changes in squamous epithelia.

Combined with the finding that expression of Ep-CAM in squamous epithelium is associated with a disturbance in the differentiation process, this suggests that Ep-CAM may actually contribute to a dysplastic/neoplastic cell phenotype by affecting the cadherin-mediated cell-cell contacts. This suggestion is in agreement with the reported disturbance in expression of integrins in CIN lesions.[25] During the normal process of keratinocyte terminal differentiation, β1-integrin expression is down-regulated.[26,27] This repression of integrin expression is an E-cadherin-controlled differentiation-related change.[27] In CIN lesions, the expression of the β1-integrin was reported in all undifferentiated/atypical cells,[25] a pattern similar to the expression pattern of Ep-CAM. In this respect, the CIN lesions resemble the structures formed *in vitro* by keratinocytes growing in the presence of anti-E-cadherin MAb.[27] If, during neoplastic changes in squamous epithelium, the *de novo* expressed Ep-CAM does indeed negatively affect cadherin-mediated junctions, one of the expected consequences would be a relief from suppression of the β1-integrin in CIN lesions, as is actually observed.

It is highly suggestive that human papillomavirus infection has a role in up-regulation of Ep-CAM in cells of the lesions in uterine cervix. Indeed, the transfection of both ecto- and endocervical epithelial cells by the human papillomavirus-16 genome results in abnormal differentiation of the cells both *in vitro* and *in vivo*.[29,30] Whether immortalization of cervical epithelial cells by human papillomavirus will indeed lead to the expression of Ep-CAM, which has to be otherwise repressed in cells of squamous differentiation, is currently under investigation.

An important consequence of our findings for immunohistology is that Ep-CAM clearly marks the neoplastic changes in squamous epithelium of the cervix. Being expressed only in areas of atypical/undifferentiated cells, Ep-CAM may serve as a good marker for grading of CINs. It can also serve as an early marker of dysplastic/neoplastic changes in cervical squamous epithelium.

References

1. Linnenbach AJ, Seng BA, Wu S, Robbins S, Scollon M, Pyrc JJ, Druck T, Huebner K: Retroposition in a family of carcinoma-associated antigen genes. Mol Cell Biol 1993, 13:1507–1515
2. Litvinov SV, Velders MP, Bakker HAM, Fleuren GJ, Warnaar SO: Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. J Cell Biol 1994, 125:437–446
3. Litvinov SV, Bakker HAM, Gourevitch MM, Velders MP, Warnaar SO: Evidence for a role of the epithelial glycoprotein 40 (Ep-CAM) in epithelial cell-cell adhesion. Cell Adhesion Commun 1994, 2:417–428
4. Litvinov SV: Ep-CAM: a homophilic cell-cell adhesion molecule with EGF-like domains. Trends Genet 1995, 7:261–271
5. Quak JJ, Van Dongen G, Brakkee JG, Hayashida DJ, Balm AJ, Snow GB, Meijer CJ: Production of a monoclonal antibody (K 931) to a squamous cell carcinoma associated antigen identified as the 17-1A antigen. Hybridoma 1990, 9:377–387
6. Tellechea O, Reis JP, Domingues JC, Baptista AP: Monoclonal antibody Ber EP4 distinguishes basal-cell carcinoma from squamous-cell carcinoma of the skin. Am J Dermatopathol 1993, 15:452–455
7. Tsubura A, Senzaki H, Sasaki M, Hilgers J, Morii S: Immunohistochemical demonstration of breast-derived and/or carcinoma-associated glycoproteins in normal skin appendages and their tumors. J Cutaneous Pathol 1992, 19:73–79
8. Smedts F, Ramaekers F, Troyanovski S, Pruszczynski M, Link M, Leigh I, Schijf C, Vooijs GP: Keratin expression in cervical cancer. Am J Pathol 1992, 141–497
9. Smedts F, Ramaekers F, Vooijs PG: The dynamics of keratin expression in malignant transformation of cervical epithelium: a review. Obstet Gynecol 1993, 82:465–474
10. Nair B, Radhakrishna P: Oncogenesis of squamous carcinoma of the uterus cervix. Int J Gynecol Pathol 1992, 11:47–57
11. Vooijs GP: Benign proliferative reactions, intraepithelial neoplasia and invasive cancer of uterine cervix. Comprehensive Histopathology. Edited by M Bibbo. Philadelphia, WB Saunders, 1988, pp 153–230
12. Levy R, Czernobilsky B, Geiger B: Subtyping of epithelial cells of normal and metaplastic human uterine cervix, using polypetide-specific cytokeratin antibodies. Differentiation 1988, 39:185–196
13. Velders MP, Litvinov SV, Warnaar SO, Gorter A, Fleuren GJ, Zurawski VR Jr, Coney LR: New chimeric anti-pancarcinoma monoclonal antibody with superior cytotoxicity-mediating potency. Cancer Res 1994, 54: 1753–1759
14. Tsutsumi K, Sun Q, Yasumoto S, Kikuchi K, Ohta Y, Pater A, Pater M: *In vitro* and *in vivo* analysis of cellular origin of cervical squamous metaplasia. Am J Pathol 1993, 143:1150–1158
15. Smedts F, Ramaekers F, Troyanovki S, Pruszczynski M, Robben H, Lane B, Leigh I, Plantema F, Vooijs GP: Basal cell keratins in reserve cells and a comparison with their expression in cervical intraepithelial neoplasia. Am J Pathol 1992, 140:601–612
16. Watt FM: Involucrin and other markers of keratinocyte terminal differentiation. J Invest Dermatol 1983, 81: 100S–103S

17. Gerders J, Lemke H, Baisch H, Wacker H-H, Scwab U, Stein H: Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki67. J Immunol 1984, 133:1710–1721
18. Inki P, Stenback F, Grenman S, Jalkanen M: Immunohistochemical localization of syndecan-1 in normal and pathological human uterine cervix. J Pathol 1994, 172: 349–355
19. Bosari S, Roncalli M, Viale G, Bossi P, Coggi G: p53 immunoreactivity in inflammatory and neoplastic diseases of the uterine cervix. J Pathol 1993, 169:425–430
20. Brown DC, Cole D, Gatter KC, Mason DY: Carcinoma of the cervix uteri: an assessment of tumor proliferation using the monoclonal antibody Ki67. Br J Cancer 1988, 57:178–181
21. Ferenczy A, Winkler B: Anatomy and histology of the cervix. Blaustein Pathology of the Female Genital Tract, ed 3. Edited by RJ Kuman. New York, Springer-Verlag, 1987, pp 141–157
22. Burghardt E: Natural history of cervical lesions. Banbury Report 2: Viral Etiology of Cervical Cancer. Edited by R Peto, zur H Hausen. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1986, pp 81–89
23. Wheelock MJ, Jensen PJ: Regulation of keratinocyte intercellular junction organisation and epidermal morphogenesis by E-cadherin. J Cell Biol 1992, 117:415–425
24. Poumay Y, Boucher F, Leclerq-Smekens M, Degan A, Leloup R: Basal cell adhesion to a culture substrate controls the polarized spatial organization of human epidermal keratinocytes into proliferating basal and terminally differentiating suprabasal populations. Epithel Cell Biol 1993, 2:7–16
25. Hughes DE, Rebello G, Al-Nafussi A: Integrin expression in squamous neoplasia of the cervix. J Pathol 1994, 173:97–104
26. Jones PH, Watt FM: Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 1993, 73:713–724
27. Hodivala KJ, Watt FM: Evidence that cadherins play a role in the downregulation of integrin expression that occurs during keratinocyte terminal differentiation. J Cell Biol 1994, 124:589–600
28. Hotchin NA, Gandarillas A, Watt FM: Regulation of cell surface β1 integrin levels during keratinocyte terminal differentiation. J Cell Biol 1995, 128:1209–1219
29. Woodworth CD, Bowden PE, Pirisi L, Barnes W, Lancaster WD, DiPaolo JA: Characterization of normal human exocervical epithelial cells immortalized *in vitro* by papillomavirus types 16 and 18 DNA. Cancer Res 1988, 48:4620–4628
30. Woodworth CD, Waggoner S, Barnes W, Stoler MH, DiPaolo JA: Human cervical and foreskin epithelial cells immortalized by human papillomavirus DNAs exibit dysplastic differentiation *in vivo*. Cancer Res 1990, 50:3709–3715